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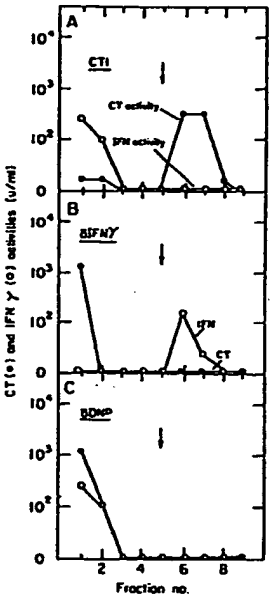
(54) A cytotoxic protein, a process for its isolation, a monoclonal antibody CT-1 and a hyrbridoma producing the same.

(57) A cytotoxic protein expressed by human peripheral blood mononuclear cells is isolated in essentially homogenous form. This protein may be used to elicit production of polyclonal or monoclonal anti-cytotoxin antibodies. Hybridomas secreting anti-cytotoxin antibodies are identified by a solid phase bioassay. The antibodies are useful in the immunopurification of cytotoxins. The purified cytotoxic proteins are useful for the treatment of virus-infected or tumor target cells, either alone or in combination with interferon or a metabolic blocker.



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Field of the invention:

There is provided in purified form a cytotoxic protein, CT, originating in human mononuclear cells. There is also provided a process for preparing such purified CT in essentially homogeneous form. There is also provided an immunoassay for the screening of hybridoma cultures in order to locate cultures producing antibodies capable of binding CT. Further, according to the invention there is provided a monoclonal antibody CT-1 specific for the CT. There is also provided a pharmaceutical composition useful for selectively treating virus infected and tumor target cells in humans which comprises CT or a salt or derivative thereof and a pharmaceutically acceptable carrier.

Background of the invention:

Proteins which exert a toxic effect on cells were found to be secreted, in response to stimulation, by mononuclear cells of various kinds. T-cells, of probably both the helper and the suppressor subsets can respond to antigens recognized by them, as well as to mitogenic lectins, by secreting such cytotoxic proteins (Granger, G.A. and Kolb, W.P., J. Imminol. 101, 111-120 (1968); Ruddle, N.H. and Waksman, B.H. J. Exp. Med. 128, 1267-1275 (1968); Eardley, D.D., Shen, F.W., Gershon, R.K. and Ruddle, N.H., J. Immunol. 124, 1199-1202 (1980)). Monocytes and macrophages produce cytotoxic proteins in response to certain bacterial toxins (reviewed by Ruff, M.R. and G.E. Gifford in Lymphotoxins, E. Pick and M. Landy editors, Academic Press, Inc. New York, 235-272, (1981)). Natural killer cells secret cytotoxic proteins upon incubation with appropriate target cells (Wright, S.C. and Bonavia, B., J. Immunol. 129, 433-439, (1982)) while cells of certain continuous B lymphocyte lines were found to produce spontaneously

cytotoxic proteins (Rosenau, W., Stites, D. and Jemtrud S., Cell. Immunol. 43, 235-244, (1979)). Proteins produced in lymphocyte cultures are usuallly referred to as "lymphotoxins", while the term "tumor necrosis factor" is often used for cytotoxic proteins produced in cultures of monocytes or of macrophages. Evidence has been presented that such cytotoxic proteins can selectively destroy tumor cells (Rundel, J.O. and Evans, C.H., Immunophormacol, 3, 9-18, (1981). So far, only a single protein of this tvpe, produced spontaneously by cells of a B lymphocyte line has been characterized in some detail. It was purified to homogeneity and its molecular weight was estimated to be about 20,000 daltons (Aggarwal, B.B., Moffat, B. and Harkins, R.N., J. Biol. Chem. 259, 686-691 (1984)).

Summary of the invention:

There is provided a purified cytotoxic protein referred to as cytotoxin (CT) and a procedure for effectively inducing this protein in monocytes or in cells derived from monocytes by virus such as Sendai virus. There is further provided a process for preparing such purified, essentailly homogeneous CT, naturally produced by peripheral mononuclear blood cells. The purified CT has a M.W. of about 17,000 daltons. CT can be isolated by the use of monoclonal antibodies against such CT which can be obtained form mice injected with partially purified or crude preparations of CT. There is provided a technique for establishing lines of lymphocytes producing such anti-CT antibodies. Such lines are advantageously established by screening a plurality of hybridomas derived from splenocytes of such immunized mice. There is also orovided a monoclonal antibody specific for CT. Such monoclonal antibody is produced by such hybridoma cell lines and is used for isolating CT in substantially homogeneous purified form.

The thus obtained purified cytotoxin, CT, is recognized by a specific anti-CT antibody. It has a M.W. of about 17,000 ± 500 daltons, as determined by analytical polyacrylamide SDS gel electrophoresis. There are also provided pharamceutical compositions useful for selectively treating virus infected cells and tumor target cells in humans which comprises CT or a salt or derivative thereof, and a pharamceutically acceptable carrier.

The term "salt" refers to salts of either or both the carboxyl and the amino groups of CT, and the term "derivatives"- to covalent modifications of the polypeptide side chains of the CT. The nature of the carrier for the CT, salt or derivative depends on the way it is applied for therapeutic purposes - be it in the form of a cream or lotion - for topical application or in the form of liquid, in which the CT, salt of derivative will be stabilized by adding components such as human serum albumin, for injection or for oral application. The purified CT is effectively cytotoxic to tumor and to virus infected cells at concentrations as low as 10 picograms/ml. The amounts of CT applied for therapy will be adjusted to reach such range of concentrations, or higher ones, in the target tissues.

The said CT is effectively cytotoxic to cells in the presence of metabolic blockers such as cycloheximide (CHI), Actinomycin D or Mitomycin C but in the absence of these agents cells of many kinds exhibit resistance to its cytotoxic effect. Infection by viruses can also render cells vulnerable to killing by the said CT. It effectively enhances, for example, the killing of VSV infected SV-80 cells while having no cytotoxic effect on uninfected SV-80 cells. Killing of virus infected cells by the said CT is potentiated by IFNs, primarily by IFN-γ, when those are applied at substantial concentrations. Thus, in

applying the said CT for therapy, pharmaceutical compositions containing also a suitable IFN are of advantage, and so are also pharmaceutical compositions containing metabolic blockers such as CHI, Actinomycin D or Mitomycin C.

Hybridoma cells producing the antibodies against CT were deposited with the International Culture Collection of Institute Pasteur, Paris, France, under Deposition No. 1-472, deposited on July 16, 1985, designated by us as Cell Line CT.

Description of the invention:

The invention is illustrated in the following by way of example.

Fig. 1 is a scheme illustrating the technique of isolation of CT. As shown the process steps involve immunizing a suitable laboratory animal (mice, etc.) with preparations enriched with CT by chromatographic procedures, followed by monitoring serum titers of CT-neutralizing and CT-binding antibodies by the techniques set out in Fig. 2a and 2b. Hybridomas derived from spenocytes of the immunized mice were screened for the production of CT-binding antibodies by the procedures set out in Fig. 3. Hybridomas found to produce such antibodies were cloned and the monoclonal antibodies produced were applied to immunoadsorbent columns on which CT was affinity-purified from preparations of lymphokines which had been induced in PBMC by concanavalin A (Con A) and phorbol-12-O-myristate 13 acetate (TPA) and then partially purified by chromatography on controlled-pore glass. The critical step was the screening of a large number of hybridoma cultures for detecting a few producing antibodies against CT. The technique developed for that purpose (set out in Fig. 3) involves a solid phase CT-binding assay, which allows a rapid screening of hybridoma cultures for the presence of such antibodies, followed by a bioassay by which CTs bound to the

solid phase can be sensitively detected, using cells sensitized to the cytotoxic effect of CT by cycloheximide.

Fig. 4. demonstrates the selectivity in the binding activity of a monoclonal antibody thus isolated, comparing CT-binding to a binding of interferon-γ by immunoadsorbents contructed form this antibody (A), as well as from two other unrelated monoclonal antibodies (B, C). It shows that of these three antibodies only one binds CT, namely, that which is directed against CT (CT-1 in Fig. 4A). It also shows that this binding of CT occurs without binding any detectable amounts of another protein in the cytotoxin preparation-IFN-γ. Under the same conditions of experiment a monclonal antibody against the latter, shown in B, does bind effectively IFN-γ without binding CT at all.

Fig. 5 shows in C the CT purified on an immunoadsorbent constructed from the monoclonal antibody, as detected by Coomassie blue staining, following electrophoresis on an acrylamide gel in the presence of SDS. (Also shown is the pattern of proteins in the crude preparation of lymphokines from which this CT has been purified, in A, the lack of any binding of protein when applying this crude preparation on an immunoadsorbent constructed from an irrelevant antibody, (against DNP) in B and molecular weight standards, in D). Fig. 6 shows how the molecular weight of this CT is estimated by comparision to the mobility, on that acrylamide gel, of the standard proteins shown in Fig, 5D. A selective cytotoxic effect of the CT and its enhancement by IFN are demonstrated in Fig. 7 which shows the cytotoxic effect of the CT at various concentrations on VSV-infected SV-80 cells (●) and its further enhancement by treating these cells with IFN-γ (10 U/ml, 16 hr prior to infection (o) or 100 U/ml. prior to infection (△)) in comparison to the resistance to CT observed in uninfected cells (■) even when they are also treated with IFN-γ at 100 U/ml (□).

Description of the preferred Embodiment:

The following example is given for illustration only.

1. Induction of CT: Human peripheral blood mononuclear cells (PBMC) are isolated on "Ficoll-Hypaque" (Pharmacia, Upsala, Sweden) from the "buffy-coats" of freshly donated blood and depleted of platelets by differential centrifugation. The cells are suspended at a concentration of 10^7 cells/ml and incubated at 37°C in MEM alpha medium (Gibco, Grand Island, N.Y.). CT is induced in these cells by one of the following techniques:

A. Preparations used for the immunization of mice are induced by stimulating PBMC with phytohemagglutinin-P (PHA). Prior to that stimulation the cells are first incubated for 12 h in the presence of a crude preparation of lymphokines (0.2 µg/ml). This treatment, does not result in the production of CT but greatly increases the responsiveness of the cells to subsequent stimulation. PHA (5 µg/ml) (Difco, Detroit) is then added and the PBMC are further incubated for 24 h. The medium is then collected, centrifuged at 2500 rpm for 15 min to remove cell debris, and processed for concentrating and enriching the CT as described below.

B. Preparations of lymphokines used for purifying CT on immunoadsorbents are advantageously induced with Con-A as it was found difficult to fully eliminate traces of PHA in the purification procedure. The cells are first treated for 12 h with 0.25 µg/ml Con-A. At this concentration Con-A does not induce significant secretion of CT but it increases the responsiveness of the cells to subsequent stimulation by a higher concentration of Con-A. Phorbol-12-O-myristate 13 acetate (TPA) is then added to a concentration of 5 ng/ml. and 3 h later Con-A us added to concentration

of 10 µg/ml. The cells are incubated for 24 h and then, following replacement with fresh media containing 5 µg/ml Con-A for a further period of 24 h. The media are combined and centrifuged, methyl α-D Mannoside (Sigma, St. Louis, Mo.) is added to a concentration of 50 mM and the media are then further processed for purification on the immunoadsorbent as described below.

C. Alternatively the CT can effectively be induced in human peripheral-blood mononuclear cells, in monocytes isolated from the mononuclear cell population or in cultured cells such as Ug37 whose properties resemble those of monocytes by applying to these cells Sendai virus (200 HAU/ml) and incubating the cells for a period of about 12 hours to allow the production of CT. The cell media are then centrifuged and processed for purification of the CT as described below.

2). Quantitation of CT: CT is quantitated by determining its cytotoxic effect by a bioassay (Wallach, D., J. Immunol. 132, 2464-2469 (1984)). Samples to be tested are applied in several serial dilutions simultaneously with the application of cycloheximide (CHI 50 µg/ml) into micro-wells containing confluent cultures of the SV-80 cells. The extent of cell killing, determined by measuring the uptake of neutral-red by the cells, is quantitated 20 hours later, by using a MicroELISA Autoreader (Dynatech, Alexandria, VA).

3. Chromatographic Enrichment of CT: Crude preparations of CT are first concentrated by adsorption to controlled pore glass (CPG) (PG-350-200 Sigma St. Louis, MO) followed by desorption in 0.5 M tetramethyl ammonium chloride (TMAC) and then further concentrated by ultrafiltration with an Amicon PM-10 membrane (Amicon, Denvers, MA). CT preparations applied for immunization of mice are then further

purified by one of the two following procedures:

(A) CPG-concentrated CT preparations are fractionated by electrophoresis on 7.5% acrylamide gels, under non-denaturating conditions (Walker, S.M. and Lucas, Z.J., J. Immunol. 113, 813-823, (1974), Lewis, J.E., Carmack, C.E., Yamamoto, R. and Granger, G.A., J. Immunol. Meth. 14, 163-176 (1977)). Fractions eluted from slices of the gels, which exhibit cytotoxic activity are pooled, concentrated by ultrafiltration on a PM-10 membrane and injected into mice.

(B) CPG-concentrated CT preparations are equilibrated with 1 M NaCl, 30% ethylene glycol, 10 mM sodium phosphate and 0.1 mM EDTA and subjected twice, sequentially, to fractionation on Ultrogel AcA44. Following each fractionation, fractions exhibiting cytotoxic activity are pooled and concentrated on a PM-10 membrane. The cytotoxic proteins recovered from the second run on the Ultrogel column are applied to further purification by preparative isoelectrofocusing on a 1% ampholine gradient (pH 3.5-10) constructed in sucrose solution using an LKB 8100-1 column. Fractions exhibiting maximal cytotoxic activity, peaking at about pH 6.4 are pooled, concentrated, equilibrated with PBS and then injected into mice.

Immunization with CT and Cell Fusion:

Four month old female CB6 mice are injected with samples of 10 µg of CT preparations - five injections with CT enriched by procedure A, as described above, and another two injections with CT enriched by procedure B. In the first immunization, the proteins are emulsified in complete Freund's adjuvant and injected into the foot pads of the mice (0.5 ml/mouse). The second injection, is given 3 weeks later, and the

rest of the injections which are given at 1 to 2 week intervals, are all given subcutaneously using alumina gel as adjuvant (0.3 µg/0.25 ml/mouse). Immunization is then discontinued for a month and the mouse showing the highest titer of serum antibodies against CT is injected twice, intraperitoneally, at a 1 day interval, with 10 µg of a CT preparation enriched by procedure B. A day after the second immunization, the mouse is sacrificed and its splenocytes are fused with myeloma cells. The fused cells are distributed into multiple wells of microtiter plates and hybridomas are selected for in HAT-containing tissue culture medium. Hybridomas found to produce antibodies against CT are cloned in soft agar. For growing these cells in the ascitic fluid of mice they are innoculated intraperitoneally at 10^7 cells per mouse 2-4 weeks following intraperitoneal injection of 0.5 ml pristan.

Quantitation of Antibodies against CT in Mouse Sera and in Hybridoma Growth Media:

The level of antibodies against CT in sera of mice is determined by measuring their neutralizing and binding activities.

CT Neutralizing Activity: (Fig. 2a)

Samples of CT (10 U in 50 µl Dulbecco's modified Eagle's medium containing 2% FCS (DMEM-2% FCS)) are incubated for 4 h at 37^{o} with samples of mouse sera (50 µl), serially diluted in DMEM-2% FCS. They are then further incubated for 12-16 h at 4^{o}C and than assayed for CT activity at eight 2-fold dilutions.

CT Binding Activity: (Fig. 2b)

Samples of crude concentrated CT (30 µl, 10^4 U/ml) are incubated for 4 h at 37^{o}C in conical-bottom micro-titer wells (Greiner) with samples of the mouse serum, serially diluted in DMEM-2% FCS. Normal

mouse serum (20 µl of a 1:40 dilution in PBS) is added, followed by 60 ul of goat antiserum against mouse $F(ab)'_2$. The plates are further incubated for 30 min at 37°C and then overnight at 4°C and are then spun at 1200 g for 5 min at 4°C. The immunoprecipitates are rinsed twice with cold PBS and once with unbuffered saline, solubilized by adding 50 µl NH_4OH and assayed for CT activity at eight 2-fold dilutions.

The Solid Phase Assay for Detecting CT Binding Monoclonal Antibodies: (applied in screening the hybridoma growth media for the presence of CT-binding antibodies, Fig. 3). PVC microtiter plates (Dynatech, Alexandria, VA) are incubated, with affinity purified goat antibody against mouse $F(ab)_2$ (80 µg/ml in PBS, 80 ul/well) then with samples of the hybridoma growth media (50 µl/well) and finally with samples of a crude concentrated CT preparation (10^4 U/ml, 50 µl/well). Each of the incubation periods is for 12-18 h (at 4°C) and following each the plates are rinsed 3 times with PBS. The plates are then further rinsed once with unbuffered saline and the bound CT is dissociated by applying NH_4OH (75 mM containing 0.1% FCS 20 µl/well). A hundred ul of 0.04 M Na-Hepes pH 7.4 in DMEM-10% FCS are added and the eluted cytotoxic activity is quantitated on CHI-sensitized SV80 cells, at four, two-fold dilutions.

Purification of CT on Immunoadsorbents:

Monoclonal antibodies are purified from ascitic fluids by precipitation with ammonium sulphate (50%). Those of the IgM isotype are further purified by dialysing against water followed by solubilitztion of the precipitating IgM in PBS. 10 mg of each of the immunoglobulins are coupled to 1 g Trisacryl GF2000 (LKB) which was derivitized with aminocaproic acid and activated with N-hydroxy succinimide. Uncoupled

antibody is removed by washing the resin with 50 mM Na-citrate pH 2.8 and then with 0.15 NH_4OH.

For purification of CT on the immunoadsorbent, samples of 0.5 ml of the resin are mixed for 2 h at 4^{o} C with 3 ml of CT preparation in the presence of 0.5 M TMAC. The resins are then packed in small columns, unbound protein is washed with 0.5 M TMAC solution. The columns are then further washed with 0.5% NP-40 in 0.5M TMAC, then with a solution of 1 M Nacl, containing also 10 mM sodium phosphate buffer pH 7.4 and then with unbuffered saline and the bound CT is eluted by applying 0.2 M NH_4OH and neutralized with 1 M acetic acid within 10 min of elution. All steps of the immunoaffinity purification procedure are carried out at 4^{o}C.

Analysis of the Purified CT by SDS Gel Electrophoresis:

Fig. 5. shows the pattern of proteins in a crude preparation of cytotoxins as analyzed on SDS-polyacylamide gel (15%). Ammonia eluted fraction from an immunoadsorbent constructed from the antibody U13-6 (against DNP) on which the crude CT has been applied (in B). CT purified from the crude preparation of the CT-1 immunoadsorbent column (in C) and molecular weight standard (phosphorylase 94K, bovine serum albumin 67K, ovalbumin 43K, carbonic anhydrase 30K, soybean trypsin inhibitor 20.1K and lysozyme 14.4K daltons (in D) as shown in Fig. 6. Fig. 5c shows that the purififed CT constitutes a single polypeptide species of U. molecular weight of the purified protein as estimated by comparison to the mobility on the acrylamide gel of other proteins with known molecular weights, is about 17.5Kd in Fig. 6.

Claims:

1. A process for the purification of a cytotoxin which comprises:
   a. providing a preparation containing a cytotoxin;
   b. absorbing the cytotoxin from said preparation onto controlled pore glass means;
   c. desorping the cytotoxin in a state of enhanced purity from said controlled pore glass means; and
   d. contacting the desorped cytotoxin with an immunosorbent, said immnosorbent comprising a monoclonal antibody against the cytotoxin.
2. The process of claim 1, where the cytotoxin is desorped in step (c) by means of a desorption buffer including 0.5M tetramethyl ammonium chloride.
3. The process of claim 1 where the cytotoxin is eluted from the immunosorbent under mild dissociating conditions.
4. The process of claim 3, where the cytotoxin is eluted from the immunosorbent by means of about 0.2M NH_4OH.
5. The process of claim 1 where the monoclonal antibody is CT-1.
6. The process of claim 1 where the cytotoxin is a human cytotoxin.
7. The process of claim 1 where the preparation of step (a) is made from stimulted peripheral blood mononuclear cells.
8. The process of claim 7 where the cells are stimulated with phytohemagglutin after incubation with lymphokines.
9. The process of claim 7 where the cells are stimulated by concanavalin-A after incubation with a concentration of concanavalin-A that does not itself induce significant secretion of cytotoxin.

10. The process of claim 7 in which the cells are stimulated with Sendai virus.

11. A solid phase immuoassay by which multiple hybridoma cultures can be screened for the production of antibodies which can bind cytotoxins, which comprises;

    a. coating protein-binding support means with affinity purified antibody against mouse immunoglobulins;

    b. Incubating the tested hybridoma growth media in the coated support means followed by washing;

    c. Incubating samples of cytotoxins in the support means followed by washing;

    d. Dissociating the cytotoxins which have bound to the support means and determining their amount in a bioassay.

12. The immunoassay of claim 11 in which said bioassay comprises providing cells sensitized to the cytoxic effect of a cytotoxin by means of a metabolic blocker, adding the cytotoxin, and measuring the extent of cell death.

13. The immunoassay of claim 12 in which the metabolic blocker is cycloheximide.

14. The immunoasay of claim 12 in which the metabolic blocker is actinomycin D.

15. The immunoassay of claim 13 in which the metabolic blocker is mitomycin C.

16. A process for preparing monoclonal antibodies against a cytotoxin which comprises immunizing mice with either pure or impure preparations of such protein, detecting hybridomas which produce such antibodies by means of the immunoassay of claim 11, cultivating such hybridomas and obtaining the desired antibodies.

17. The process of claim 16, in which said bioassay comprises providing cells sensitized to the cytotoxic effect of a cytotoxin by means of a metabolic blocker, exposing the cells to the cytotoxin, and measuring the extent of cell death.
18. The process of claim 17 in which the cells used in the bioassay are cycloheximide-sensitized SV80 cells.
19. A monoclonal antibody specific for cytotoxins
20. A monoclonal antibody CT-1 which specifically recognizes and binds human cytotoxin, said cytotoxin being produced by peripheral blood mononuclear cells.
21. A process for isolation of a cytotoxin which comprises:
    a. Developing monoclonal antibodies against such cytotoxin, following immunization with impure preparations of these cytotoxins;
    b. Constructing an immunoadsorbent from these antibodies and using the same for purifying the cytotoxin, from crude preparations of thereof.
22. A pharmaceutical composition for selectively treating virus-infected and tumor target cells in humans, which comprises a therapeutically effective amount of at least one cytotoxin, or salt thereof in essentially homogenous form, or in combination with interferons and/or a metabolic blocker in amount sufficient to sensitize said cells to said cytotoxin but not to sensitize normal cells.
23. The composition of claim 22 where the blocker is cycloheximide.
24. The composition of claim 22 where the blocker is mitomycin C
25. The composition of claim 22 where the blocker is actinomycin D.
26. A hybridoma cells line expressing a monoclonal antibody against a cytotoxin.

27. The hybridoma cell line of claim 26, having the identifying characteristics of cell line CT, CNCM I-472.

28. A monoclonal antibody specific for a cytotoxin, obtained by the process of claim 16.

29. A monoclonal antibody specific for a cytotoxin, obtained by the process of claim 17.

30. A process for the purification of a cytotoxin which comprises contacting a preparation containing a cytotoxin with an immunosorbent, said immunosorbent comprising a monoclonal antibody against the cytotoxin.

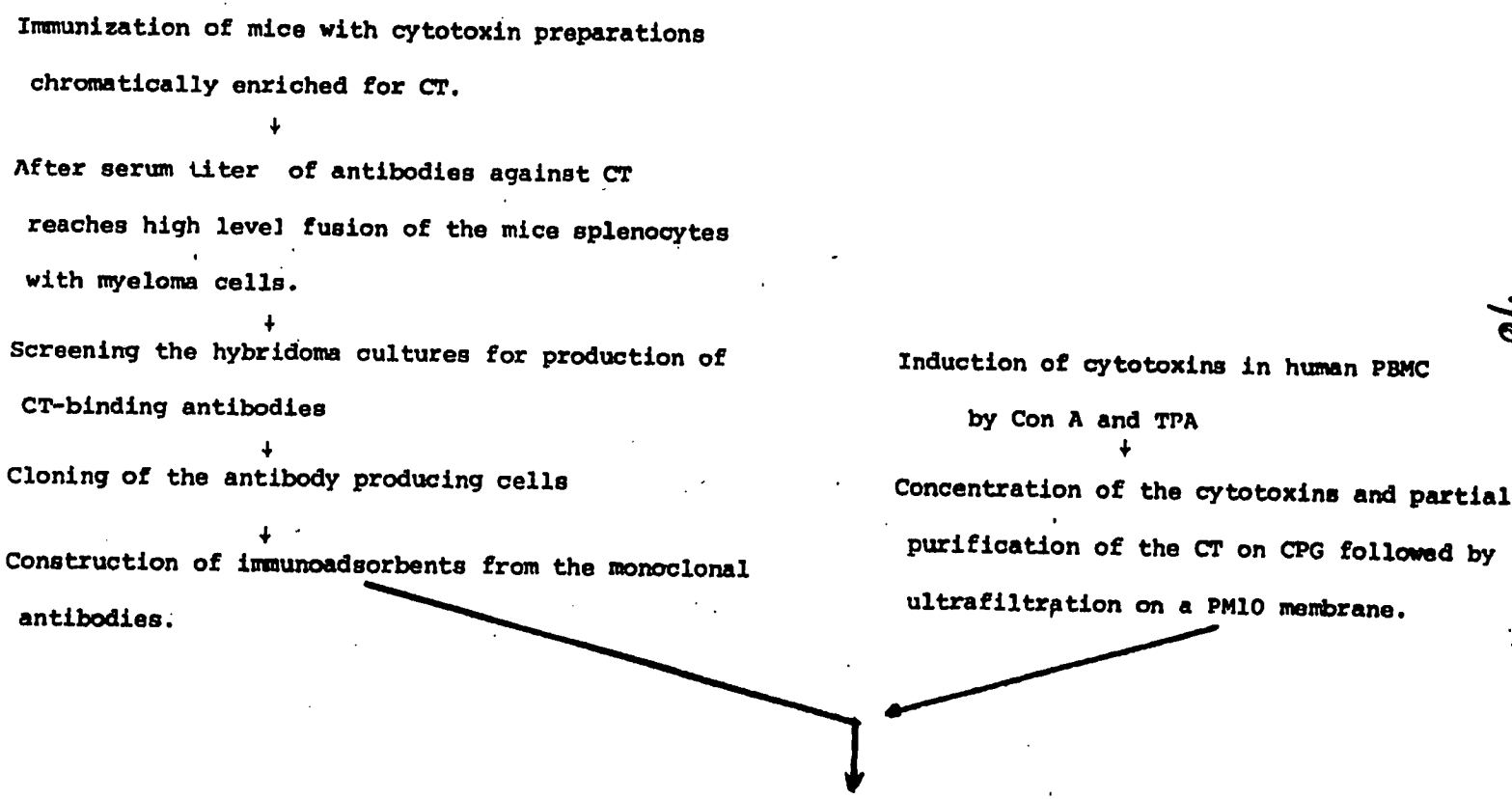

Fig. 1

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Alternative Approaches for Detection of Antibodies against CT in Sera of Immunized Animals

a Neutralization assay:	Incubation of CT preparation with antiserum.	→ Determination of CT activity (in the presence of antiserum)		
b Binding assay:	Incubation of CT preparation with antiserum	→ Precipitation of serum immunoglobulin+bound CT with goat antiserum against mouse immuno-globulins.	→ Washing of immuno precipitate followed by its solubilization at 0.075M ammonia	→ Determination of T activity in solubilized immunoprecipitate

Fig. 2

The Solid Phase Assay for Detecting, CT Binding, Monoclonal Antibodies

Adsorbtion of hybridoma produced immunoglobulin to PVC microwells which had been precoated with affinity purified antibodies against mouse immunoglobulins. → Incubation of CT preparations in microwells. → Rinsing of microwells followed by dissociation of bound antigens at 0.075M ammonia → Determination of α activity in the proteins eluted from microwells

Fig. 3

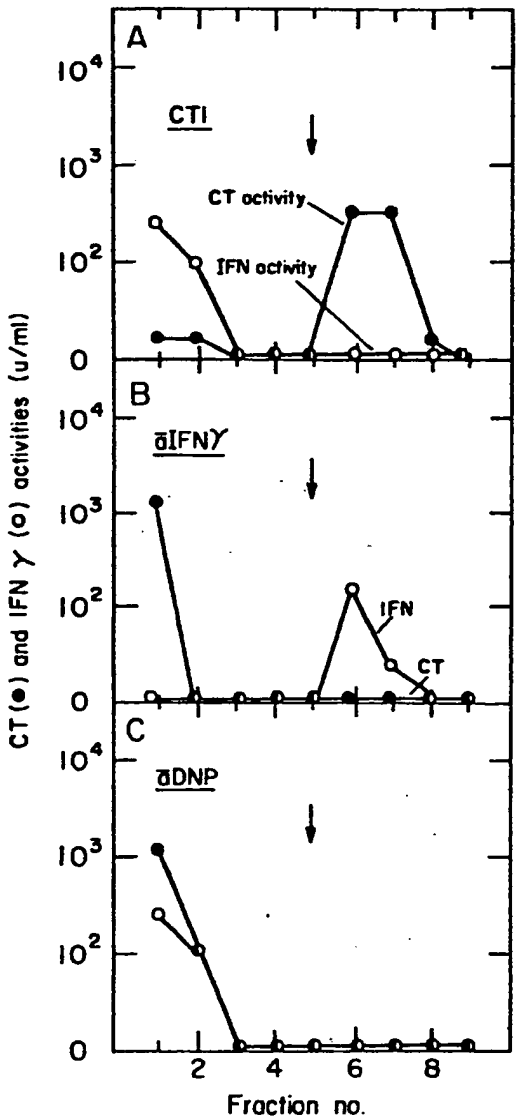
A
CTI
CT activity
IFN activity
B
αIFNγ
IFN
CT
C
αDNP
CT (●) and IFN γ (○) activities (u/ml)
Fraction no.

FIG 4

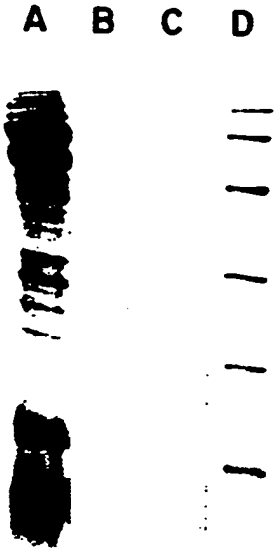
A
B
C
D

FIG 5

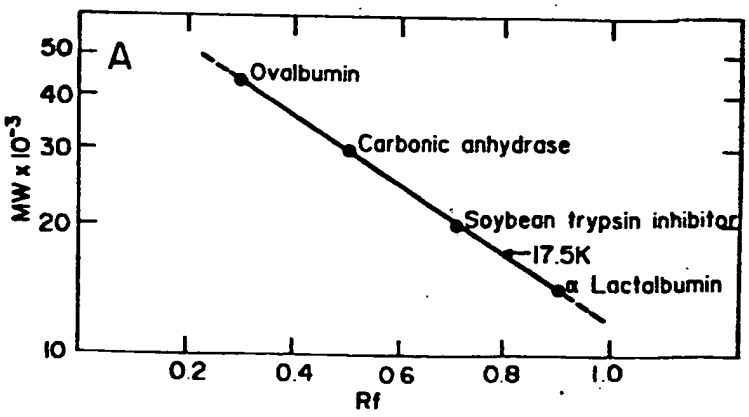
A
Ovalbumin
Carbonic anhydrase
Soybean trypsin inhibitor
17.5K
α Lactalbumin
MW x 10⁻³
50
40
30
20
10
0.2
0.4
0.6
0.8
1.0
Rf

FIG 6

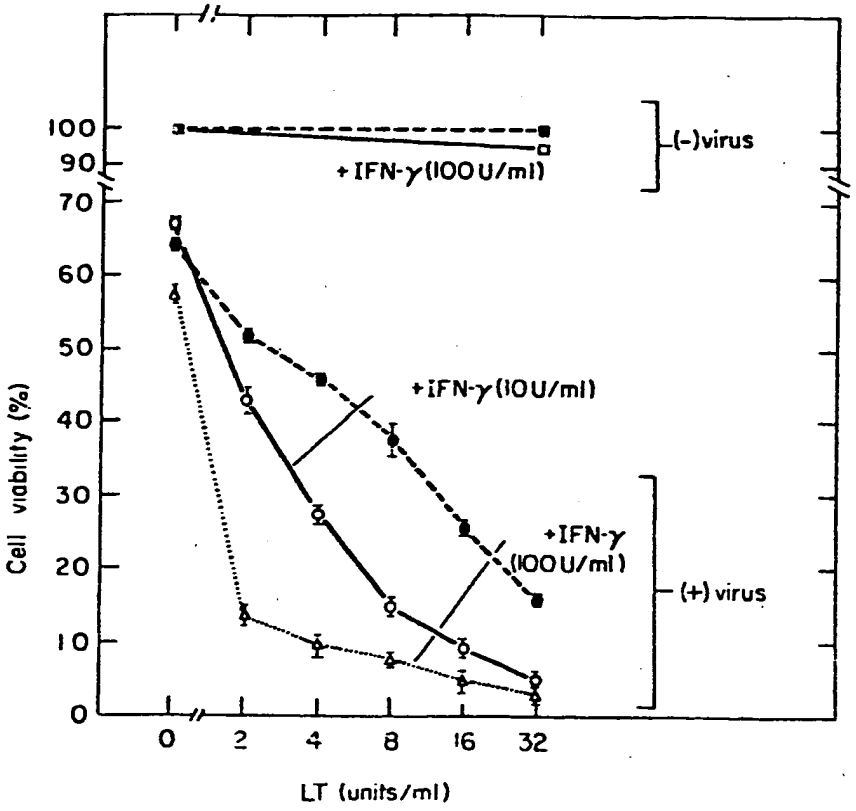
100
90
70
60
50
40
30
20
10
0
Cell viability (%)
+IFN-γ(100U/ml)
(-)virus
+IFN-γ(10U/ml)
+IFN-γ
(100U/ml)
(+) virus
0
2
4
8
16
32
LT (units/ml)

FIG. 7.